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Diurnal and circadian patterns of gene expression in the developing xylem of *Eucalyptus* trees

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Abstract

The daily cycle of night and day affects the physiology and behaviour of almost all living things. At a molecular level, many genes show daily changes in expression level, often providing adaptive benefit to the organism. The roles that such daily patterns of gene expression play in coordinating carbon allocation processes during wood development in trees are not fully understood. To identify genes with diurnal expression patterns in wood-forming tissues of field-grown *Eucalyptus* trees, we used a cDNA microarray to measure transcript abundance levels at roughly four-hour intervals throughout a diurnal cycle in two clonal hybrid eucalypt genotypes. Eight percent of genes on the microarray (217 out of 2608) exhibited diurnally influenced expression profiles. Affected genes included those involved in carbon allocation, hormone signalling, stress response and wood formation. *Eucalyptus* homologues of the central clock genes *Circadian Clock Associated 1 (CCA1) and GIGANTEA (GI)* were expressed in developing xylem tissues and cycled with a circadian rhythm in constant light. The presence of a functional biological clock and diurnal transcript abundance patterns during xylogenesis suggests important roles for temporal control of xylem development and metabolism in fast-growing plantation trees.

Keywords: *Arabidopsis*; Carbon allocation; Microarray; Wood formation

1. Introduction

Many metabolic processes in plants are directly or indirectly influenced by the photoperiod, as temporal changes in light and temperature lead to diurnal rhythms in physiology (McClung, 2006). A subset of these rhythms is endogenously driven by the circadian clock, in an evolutionary adaptation to the daily rhythm of night and day (Pittendrigh, 1960). The circadian clock is an internal biochemical oscillator with a period of approximately 24 h. It enables the anticipation of daily changes in the environment, and provides adaptive benefit in plants through enhanced photosynthesis, carbon fixation and growth (Dodd et al., 2005a,b).

Trees, like other plants, show diurnal and circadian rhythms in physiology and gene expression that influence growth and development (Rogers et al., 2005; Böhlenius et al., 2006). Primary metabolism is directly influenced by diurnal changes in the availability of light for photosynthesis. Daily rhythms in gene expression and metabolite abundance are therefore important in coordinating carbon supply and utilisation (Stitt et al., 2007). Secondary metabolic pathways are also affected by daily rhythms. This is the case for phenylpropanoid biosynthesis and lignin production which are both temporally controlled (Harmer et al., 2000; Rogers et al., 2005). Long lasting changes, such as seasonal growth, dormancy and flowering which occur over months or years are also controlled by the circadian clock. These processes are crucial for the survival and adaptation of woody perennials (Ramos et al., 2005; Böhlenius et al., 2006).

The circadian system, including transcriptional networks and temporally controlled biological processes are largely conserved between the well described *Arabidopsis* (Harmer et al., 2000;

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Edwards et al., 2006; Covington and Harmer, 2007) and poplar systems (Michael et al., 2008). This functional conservation is likely to extend to other plants. For example, orthologues of *Arabidopsis* clock genes have been identified in chestnut trees (*Castanea* spp.) and their mRNA abundance was found to cycle circadianly (Ibañez et al., 2008). In contrast to the well described circadian system in *Arabidopsis*, much remains to be learnt about the circadian and diurnal control of tree genes, their biological function and level of regulation.

Eucalyptus tree species constitute the most extensively planted hardwood crop in the world due to their fast growth, superior wood properties and wide adaptability (Eldridge et al., 1994). Fast-growing *Eucalyptus* trees have also been earmarked as future bioenergy crops for the production of cellulosic biofuels (JGI, 2007). To develop trees with improved wood and fibre properties for bioprocessing, a comprehensive understanding of tree growth and development is required. An important aspect of this is describing the dynamics of carbon allocation to various sink organs of the tree, and to the different components of the plant cell wall. Woody plants stand to benefit greatly from circadian coordination, due to the distance between their sink and source organs and their long life spans which encompass multiple changes of season. Temporal coordination of wood formation and carbon allocation pathways in trees could lead to enhanced energy efficiency and improved growth.

This study was undertaken to determine the prevalence and potential roles of diurnally and circadianly regulated transcripts abundantly expressed during wood formation in *Eucalyptus* trees. The study identified 217 genes with quantitative changes in transcript abundance over the course of a diurnal cycle in field-grown trees. These genes were involved in wood formation, carbon allocation and stress responses. Their expression patterns may reveal new insights into temporal regulation of these biological processes in woody plants.

2. Materials and methods

2.1. Sampling of plant material

Tissue samples for a diurnal time series were collected over three days in early spring (August) from a field-trial of two three-year-old hybrid *Eucalyptus* clones (*Eucalyptus grandis* W. Hill ex Maid. × *E. camaldulensis* Dehnh., 'GC' and *E. grandis* × *E. urophylla* S.T. Blake, 'GU,' Sappi Forests). These hybrids are widely planted due to their superior drought and disease tolerance over pure species *E. grandis* (Eldridge et al., 1994; Retief and Stanger, 2007). The field-trial was situated near KwaMbonambi in sub-tropical northern KwaZulu-Natal, South Africa. Soft, non-fibrous differentiating xylem tissue of standing trees was collected by peeling the bark off the stems from one to two meters above ground level, and lightly scraping the exposed xylem tissues. Stems were debarked in sections to avoid wound-related gene expression and samples from the entire circumference of the trunk were bulked to avoid positional effects. Samples were collected at approximately four-hour intervals from one GC and one GU ramet per time point, and thereafter treated as biological replicates. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Differentiating xylem tissue was also collected from potted ramets of the same genotypes as the field-grown trees. Potted ramets were grown outdoors until they reached approximately 1.5 m in height, before being moved into a growth room under controlled light conditions. Plants were entrained to a 12 h light/12 h dark cycle (LD) at a light intensity of 100 μmol m^{-2} s^{-1} for three weeks, after which sampling began at 06:00 (first light), continuing for 72 h at six-hourly intervals. Growth conditions were switched to continuous light (LL) at dawn on the second day of sampling. Sampling followed the model of the field-grown trees on a smaller scale: stems were severed at ground level, bark removed from approximately one centimetre to one meter above ground level and a scalpel blade used to scrape immature xylem tissue from the stem. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Experimental design, target preparation and microarray analysis

Two separate microarray experiments were performed using RNA extracted from each of the biological replicates (field-grown GU or GC trees). A loop design was used for each of the two replicates, linking six time points extending from 06:00 on day two to 02:00 on day three (Fig. 1). A dye swap was incorporated in each experiment as a technical replicate to eliminate dye bias. The loop design allowed transcript abundance at each time point to be compared to all other time points.

Total RNA was isolated from woody tissues using the cetyl trimethylammonium bromide (CTAB) based method of Zeng and Yang (2002) with the following adaptations: for field-grown samples, 20 ml of extraction buffer were used with four grams of ground tissue; for samples from potted plants, one gram of tissue was extracted with 10 ml of buffer. Field-grown tissue samples were ground to a fine powder in liquid nitrogen using a high-speed grinder (IKA-Werke, Staufen, Germany). Potted plant samples were ground using a mortar and pestle with liquid nitrogen. All centrifugation steps were performed at 12,000 × *g*. Isolated total RNA was purified using the RNeasy kit (Qiagen Inc., Valencia, CA) and analysed for purity and quantity on a Nanodrop spectrophotometer (Nanodrop Technologies ND 1000, DE, USA) and by 1% agarose gel electrophoresis.

Aminoallyl-labelled cDNA was prepared for hybridisation to microarray slides by reverse transcription from 15 μg total RNA, using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) primed with 1 × random hexamer primers (Roche Diagnostics). cDNA was purified and labelled with either Cy3 or Cy5 dye (Amersham Biosciences, Piscataway, NJ), and excess dye molecules removed using a PCR Purification Kit (Qiagen Inc., Valencia, CA) according to The Institute for Genome Research (TIGR) protocol #M004 (http://compbio.dcfi.harvard.edu/docs/MicrarrayLabeling.pdf). Dye incorporation was measured using a Nanodrop spectrophotometer. The two samples for each slide were combined so that each contributed an equal quantity of dye. Mixed samples were dried in a vacuum centrifuge (ThermoSavant VLP80, NY, NY), re-suspended in 60 μl of a

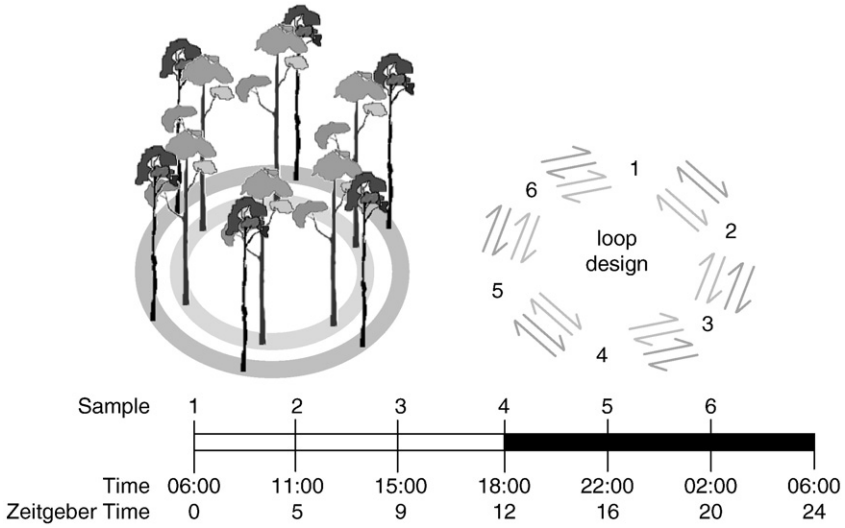

Fig. 1. Experimental design: immature xylem was harvested at approximately four-hour intervals from the stems of three-year-old ramets of a *E. grandis* × *E. urophylla* (GU) and an *E. grandis* × *E. camaldulensis* (GC) clone at the times indicated. Transcript profiles were compared in two separate experiments (GU and GC) using a loop design linking the six sampled time points for each genotype. Each arrow represents a microarray slide, the sample at the head of the arrow being labelled with Cy5 and the tail with Cy3.

1 × hybridisation buffer (50% formamide, 5 × Saline Sodium Citrate (SSC), 0.5% Sodium Dodecyl Sulfate (SDS), 5 × Denhardt's Solution, 0.5 μg/μl Poly d(A) and 0.5 μg/μl calf thymus DNA), denatured for 3 min at 95 °C and cooled on ice before applying to the pre-hybridised slide and covered with a cover-slip.

Microarray slides were pre-hybridised in a 1 × pre-hybridisation buffer (5 × SSC, 1% Bovine Serum Albumin (BSA), 0.1 × SDS) for 45 min at 42 °C, rinsed twice in Ultra High Quality H_2O; once in isopropanol, and dried by centrifugation. Prepared slides were placed in hybridisation chambers and incubated for 18–20 h at 42 °C in a water bath. Post-hybridisation washes were performed in three wash solutions of increasing stringency (Wash 1: [1 × SSC, 0.2% SDS at 42 °C]; Wash 2: [0.1 × SSC, 0.2% SDS at room temperature]; Wash 3: [0.1% SDS at room temperature]). Washes 1 and 2 were performed once each while Wash 3 was performed three times. Slides were dried by centrifugation and scanned within the hour.

A spotted cDNA array previously described by Kirst et al. (2004) was used. Briefly, the array consisted of 2608 cDNA fragments derived from *E. grandis* and *E. tereticornis* cDNA libraries. Spots were replica printed four times on each microarray slide (Corning Gap II, Corning, NY, supplied by ArrayXpress, Raleigh, NC, USA).

2.3. Data treatment and statistical analysis

Slides were scanned on a Genepix™ 4000B scanner (Axon Instruments, Foster City, CA) at the ACGT Microarray Facility (www.microarray.up.ac.za). Laser and photomultiplier settings were adjusted to obtain a signal ratio of 1:1 between the two dyes. Image analysis was performed with GenePix Pro 5.0 (Axon Instruments, Foster City, CA). Raw signal intensity values were transformed ($\log_2$) and the four replicate spots for each target on a single slide were averaged. The transformed and averaged data were then imported to JMP Genomics V2.04 (SAS Institute, Cary, NC) for global normalisation and gene modelling. First, a mixed model analysis of variance (Wolfinger et al., 2001) was performed to account for experiment-wide variation associated with 'time', 'array' and 'dye' effects as well as 'array' × 'dye' interaction. Residual values from this model were then used as inputs for global Local Estimate of Scatterplot Smoothing (LOESS) normalisation.

The normalised residuals were analysed in a second, gene-specific, mixed model ANOVA, where the effect of 'time' on the expression of every gene was analysed individually. *P*-values were calculated for the effect of 'time' on every gene, within and across genotypes. A gene was considered to be differentially expressed (diurnally responsive) if the effect of 'time' was significant in at least one of the time points relative to all other time points ($-\log_{10}P > 3.74$). A False Discovery Rate (FDR) of $\alpha = 0.01$ was used to correct multiple testing. Data collected from both genotypes were considered together as biological replicates to calculate least square means (LSM) estimates of gene expression. This was done in order to identify conserved components of diurnal and circadian regulation in *Eucalyptus*. Raw and normalised data from the microarray experiment were submitted to the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, series number GSE11731).

2.4. Quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis

qRT-PCR reactions were performed with the LightCycler® 480 instrument and LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannhein, Germany) according to the manufacturer's instructions. PCR reactions were carried out in triplicate, and amplification of a single product confirmed by melting curve analysis and agarose gel electrophoresis. Raw qPCR data quality control; gene-specific PCR efficiency correction, and multiple reference-gene normalisation were performed

using qBase software (Hellemans et al., 2007). qBase uses an adapted delta–delta–Ct method to calculate relative expression. Graphs were generated in Microsoft Excel using the 'smoothed line' option. Gene-specific primers for qRT-PCR analysis were designed using Primer Designer 4 (Scientific and Educational Software, Cary, NC). All primer sequences are provided in Table S1.

2.5. Gene ontology

Functional categorisation of all genes on the microarray slide was performed on TAIR (The *Arabidopsis* Information Resource: www.arabidopsis.org/tools/bulk/go/index.jsp) using the 'functional categorisation' option which groups genes into broad functional categories based on high level terms in gene ontology (GO) hierarchy. Statistical significance of GO term enrichment in the diurnally responsive set was assessed using FuncAssociate (http://llama.med.harvard.edu/Software.html), which computes the probability of finding a certain number of significant genes given the starting list, using Fisher's Exact Test (Berriz et al., 2003).

3. Results

3.1. Microarray expression profiling in field-grown trees

To identify diurnally influenced wood formation and carbon allocation genes, we sampled developing xylem tissues at four-hourly intervals from clonal ramets of two related genotypes of field-grown *Eucalyptus* trees. Labelled cDNA derived from total RNA collected at six consecutive time points (Fig. 1) was used to probe the microarray slides. The array included 2608 gene fragments from *Eucalyptus* xylem, leaf, petiole, root and flower cDNA libraries (Kirst et al., 2004). Expression levels across the six time points were compared using a microarray loop design incorporating a dye swap and considering the two related genotypes as biological replicates (Fig. 1).

A total of 217 cDNA targets were significantly differentially expressed at one or more of the six time points in either one (96/217) or both (113/217) of the genotypes. The eight remaining targets had significant but opposite profiles in the two genotypes and were not significant in the joint analysis. Significance levels were determined based on a False Discovery Rate of 0.01, corresponding to a $-\log_{10}P$ value of at least 3.74 at one or more of the six time points. The differentially expressed gene set had a range of expression ratios from 2.7-fold to over 30-fold change over a diurnal cycle (results not shown). Standardised LSM intensity values were calculated using data from both genotypes to visualise their shared expression patterns (Fig. 2).

The 217 targets represented 8.3% of the genes on the array and corresponded to 195 unique BLAST hits to *Arabidopsis* gene IDs. Thirty-six of the 195 unique gene models (18.5%, Table 1 marked *) were circadianly regulated in *Arabidopsis* according to a previous genome-wide study in the model plant (Covington and Harmer, 2007). Peak and trough expression times for the 217 targets were distributed across all time points, with Zeitgeber Time 16 (ZT 16, 22:00) being best represented for peak expression (61 genes) and ZT 12 (18:00) having the fewest genes peaking (12 genes, Fig. S1a,b). Zeitgeber Time measures hours since the last dark/light switch. Sunrise occurred close to 06:00 at the field sampling location, giving a day length of approximately 12 h. The majority of genes on the microarray did not show significant diurnal changes in expression (Fig. S1c).

The 217 cDNA targets showing significant diurnal variation were clustered based on the similarity of their expression patterns (Fig. 2a), and 10 broad gene expression clusters were defined (Fig. 2b). Each cluster showed a distinct expression profile, containing either one or two daily peaks in expression.

3.2. Confirmation of microarray transcript abundance profiles by qRT-PCR

To confirm the accuracy of the microarray results, the expression levels of five genes selected from the microarray were profiled using qRT-PCR analysis. The same tissue samples profiled in the microarray experiment were used for the qRT-PCR profiling. Normalised qRT-PCR expression profiles of the five test genes closely followed those determined by the microarray analysis (Fig. S2). The *Eucalyptus* homologue of the *Arabidopsis* central clock oscillator gene Circadian Clock Associated 1 (*CCA1*) (Fig. S2e) had a diurnal expression pattern in *Eucalyptus* xylem, closely matching that of *CCA1* in *Arabidopsis* with a morning peak in expression (Wang and Tobin, 1998), further supporting the microarray results.

3.3. Functional characterisation of diurnally responsive genes

The diurnally responsive gene set were representative of many different metabolic pathways (Table 1). To determine whether any molecular function or subcellular compartment was over-represented in the diurnally responsive set, we classified all significant genes by their associated GO terms according to TAIR (www.arabidopsis.org). The proportion of diurnally responsive genes found in each GO class was contrasted with that of all genes present on the microarray slide. The diurnal set was most enriched relative to the whole slide, in genes from the cytosol, cell wall and ribosome compartments. Response to stress, along with response to abiotic/biotic stimulus were the functions with the largest proportional changes in representation (Fig. 3).

FuncAssociate, a web-based tool used to characterise gene sets by GO attributes and determine significant differences (Berriz et al., 2003) showed 'response to stress,' incorporating 'response to temperature' (GO Attributes 0006950 and 0009266) to be significantly over-represented ($P = 1.2 \times 10^{-4}$, Fig. 3). Included among the stress response genes were several coding for heat shock proteins (Table 1).

Twenty-eight of the diurnally responsive genes were likely to be involved in starch/sucrose metabolism (Table 1), including genes involved in the production and modification of cellulose and hemicellulose. Fourteen of these were classified as carbohydrate active enzymes, or CAZymes (Geisler-Lee et al., 2006). Of the 14 CAZymes found to be diurnally influenced in

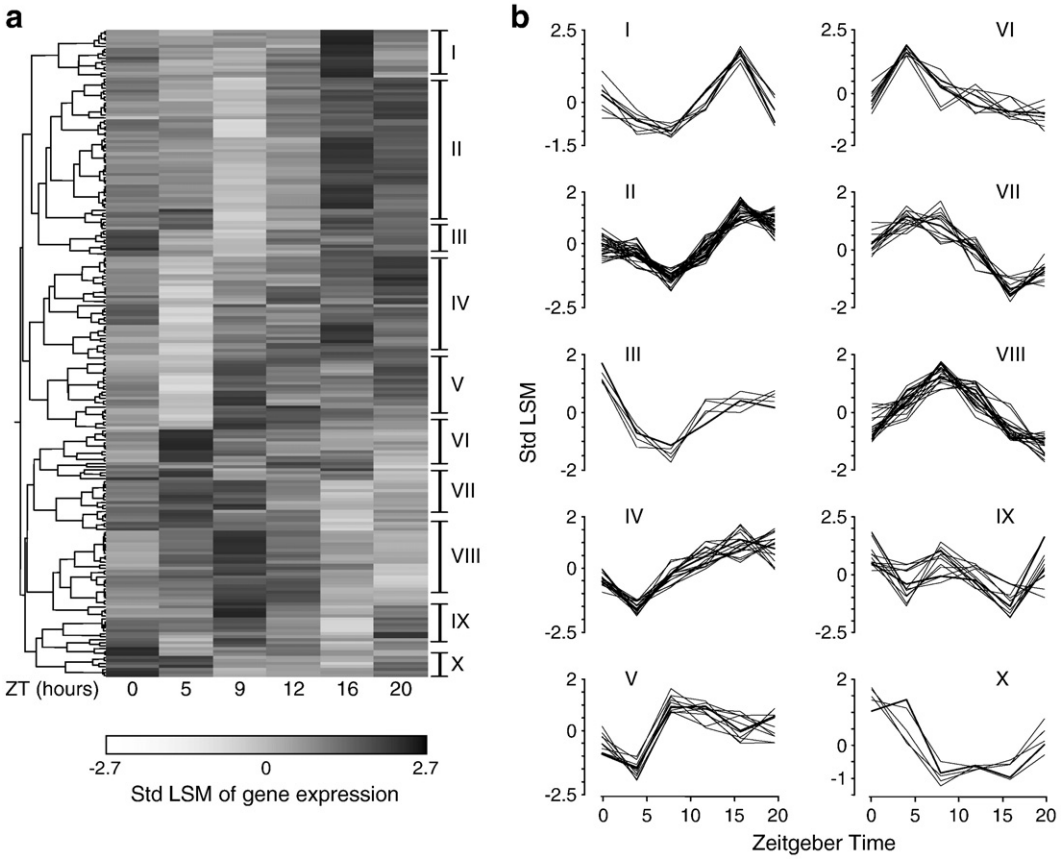

Fig. 2. Clustering of diurnally affected microarray gene expression profiles. (a) Hierarchical clustering of 217 differentially expressed microarray targets. Relative expression levels (Std LSM: standardised least square means) are represented by a greyscale continuum, with white indicating lowest expression, and black highest. Rows represent individual microarray targets, columns represent sampling time points. Ten groups sharing similar expression profiles are indicated by vertical bars labelled 'I' to 'X' to the right of the cluster. (b) Line graphs of representative genes for clusters I–X showing the dominant profile of each group.

Eucalyptus xylem, five were glycosyl transferases (GT), eight were glycosyl hydrolases (GH), and one was a carbohydrate esterase (CE, Table 1).

3.4. Expression profiling of central clock homologues in Eucalyptus xylem

The expression profiles of three *Eucalyptus* genes showing homology to known *Arabidopsis* clock genes were analysed. The *Eucalyptus* homologue of the central clock gene *CCA1* was identified as diurnally expressed by our microarray results. The cDNA represented by the microarray feature (GenBank Acc: CD668918) showed similarity to the CCA1 protein of *Arabidopsis* (At2g46830, BlastX $E=1\times10^{-8}$). The second central clock gene was not present on the microarray slide, but was previously found to be expressed in *E. grandis* xylem (Ranik et al., 2005). The sequence (GenBank Acc: DN596733) showed strong similarity to a central clock protein GIGANTEA (GI) from *Arabidopsis* (BlastX $E=3\times10^{-20}$). The *Eucalyptus* homologue of the *Arabidopsis* clock-related gene *Zeitlupe* (ZTL) was also identified as a diurnally expressed gene from the microarray results. Its corresponding microarray probe (GenBank Acc: CD668500) showed strong similarity to the ZTL protein of *Arabidopsis* (At5g57360, BlastX $E=4\times10^{-53}$). The three *Eucalyptus* clock gene homologues are herein referred to as *EtCCA1*, *EtZTL* and *EgGI,* to indicate their origins.

To confirm the expression and phase of *EtCCA1* and *EgGI* in *Eucalyptus* xylem, we profiled their expression patterns in the GU and GC clones using qRT-PCR. Immature xylem samples from the field-grown trees and the potted ramets grown in controlled light conditions were examined. Cosine shaped expression profiles were observed in the mRNA levels of both genes in light/dark conditions (LD) as well as in constant light (LL), indicating the endogenous nature of their regulation (Fig. 4). The expression patterns of *EtCCA1* and *EgGI* matched those of their *Arabidopsis* counterparts (Wang and Tobin, 1998; Fowler et al., 1999; Alabadi et al., 2001) with *EtCCA1* expression peaking at dawn (ZT 0) and *EgGI* peaking between midday and dusk (ZT 6-12).

EtZTL mRNA abundance showed one of the largest diurnal changes of all genes on the microarray (26-fold, results not shown). The expression fluctuation of the gene had a cosine shaped profile which peaked at ZT 9 (15:00, Fig. S3), approximately the same time as *EgGI*.

3.5. Metabolic pathway analysis

The microarray expression data were grouped according to metabolic pathways to enable visualisation of diurnal influence

Table 1
Putative identities (BlastX best hit against *Arabidopsis* proteins) and time of highest expression (↑) of diurnally influenced *Eucalyptus* genes in pathways of interest.

Eucalyptus EST	At number[a]	Description	CAZyme family[b]/category[c]	06:00	11:00	15:00	18:00	22:00	02:00
(A) Circadian clock-related									
eef1c.pk001.i8	At2g46830	Myb-related transcription factor (CCA1) *	TF	↑					
eec1c.pk003.d5	At5g57360	Zeitlupe (ZTL)				↑			
(B) Starch/sucrose metabolism									
eef1c.pk005.e20	At1g06410	Putative trehalose biosynthesis enzyme		↑					
eef1c.pk002.a17	At1g08200	UDP-D-glucuronic acid decarboxylase						↑	
eej1c.pk004.e12	At1g14720	Xyloglucan endo-transglycosylase	GH16[b]					↑	
eef1c.pk005.b4	At1g19170	Pectinase	GH28[b]			↑			
eep1c.pk005.l14	At1g60470	Galactinol synthase	GT8[b]/stress	↑					
eep1c.pk005.e16	At2g21730	Mannitol dehydrogenase				↑			
eef1c.pk002.l4	At2g28760	UDP-D-glucuronic acid decarboxylase							↑
eec1c.pk004.e1	At2g36460	Fructose bisphosphate aldolase						↑	
eej1c.pk001.l5	At2g36530	Phosphopyruvate hydratase							↑
egx20f08	At2g45290	Transketolase							↑
eec1c.pk001.n22	At3g04120	GAPDH				↑			
eef1c.pk003.d12	At3g15020	Malate dehydrogenase		↑					
eea1c.pk005.b13	At3g18080	Beta-glucosidase	GH1[b]			↑			
eea1c.pk005.p23	At3g21560	UDP-glucosyltransferase *						↑	
egx28e09	At3g23920	β-Amylase *	GH14[b]/stress		↑				
eej1c.pk002.g13	At3g43190	Sucrose synthase	GT1[b]						↑
eef1c.pk004.d20	At3g52930	Fructose bisphosphate aldolase					↑		
eec1c.pk006.c12	At3g52990	Pyruvate kinase				↑			
eea1c.pk002.h11	At3g55700	UDP-glucosyl transferase family protein	GT1[b]			↑			
eea1c.pk006.h24	At4g02290	Endo-1-4-β-glucanase	GH9[b]		↑				
eep1c.pk003.f22	At4g17770	Putative trehalose biosynthetic enzyme				↑			
egx28b09	At4g19410	Pectinacetylesterase	CE13[b]					↑	
eec1c.pk001.m22	At5g20830	Sucrose synthase	GT1[b]						↑
egx19g03	At5g49720	Endo-1-4-β-glucanase	GH9[b]			↑			
eep1c.pk005.e3	At5g59290	UDP-D-glucuronic acid decarboxylase						↑	
eef1c.pk004.h1	At5g64790	Glycosyl hydrolase family 17 protein	GH17[b]		↑				
eep1c.pk004.f23	At5g66460	Endo-1-4-β-glucanase	GH5[b]					↑	
egx22b01	At5g67230	Glycosyl transferase family 43 protein	GT43[b]			↑			
(C) Calcium signalling-related									
eej1c.pk002.l21	At1g62820	Calmodulin, contains calcium-binding domain						↑	
eer1c.pk001.o22	At2g02010	Glutamate decarboxylase (GAD1)	Stress					↑	
eea1c.pk002.d4	At4g27280	Calcium-binding EF hand family protein			↑				
(D) Lignin biosynthesis									
eej1c.pk004.j8	At1g72680	Cinnamyl alcohol dehydrogenase (CAD)				↑			
eec1c.pk004.c1	At2g30490	Cinnamate-4-hydroxylase (C4H) *				↑			
eea1c.pk007.f7	At2g40890	Coumarate-3-hydroxylase (C3H) *					↑		
eec1c.pk003.m8	At3g19450	Cinnamyl alcohol dehydrogenase (CAD) *				↑			
eec1c.pk002.f5	At4g34050	Caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT)						↑	
eep1c.pk001.i12	At4g36220	Ferulate-5-hydroxylase (F5H)				↑			
(E) Water transport									
eec1c.pk002.g8	At3g16240	δ-Tonoplast intrinsic protein (δ-TIP)						↑	
eej1c.pk005.l13	At3g53420	Plasma membrane intrinsic protein 2B (PIP2B) *				↑			
(F) Transcription factor (TF)									
eep1c.pk001.c21	At1g66230	Putative transcription factor (ATMYB20)				↑			
eef1c.pk006.i22	At2g22800	Homeobox-leucine zipper protein 9 (HAT9)					↑		
egx28c07	At2g46680	*Arabidopsis thaliana* Homeobox 7 (ATHB7)	Stress		↑				
eej1c.pk005.e22	At5g04760	Myb family transcription factor				↑			
egx20a08	At5g22570	WRKY transcription factor 38							↑
(G) Stress response									
eea1c.pk004.k20	At1g08830	Copper/zinc superoxide dismutase			↑				
eef1c.pk005.c1	At1g20030	Pathogenesis-related thaumatin family protein				↑			
eea1c.pk004.b17	At1g46264	Heat stress transcription factor family member	TF	↑					
egx06h08	At1g52560	26.5 kDa class I small heat shock protein-like					↑		
egx20a06	At1g54050	17.4 kDa class III heat shock protein			↑				

Table 1 (*continued*)

Eucalyptus EST	At number[a]	Description	CAZyme family[b]/category[c]	06:00	11:00	15:00	18:00	22:00	02:00
(G) Stress response									
eec1c.pk006.m17	At1g56070	Cold-induced translation elongation factor						↑	
eej1c.pk006.l24	At1g69450	Early-responsive to dehydration (ERD 15)						↑	
egx06b05	At2g18150	Peroxidase				↑			
egx25e11	At2g41430	Dehydration-induced protein (ERD 4)*						↑	
eea1c.pk007.n4	At3g09640	L-Ascorbate peroxidase 1						↑	
eec1c.pk006.k23	At3g12580	Heat shock protein 70*				↑			
eef1c.pk002.h5	At3g12610	DNA-damage repair/toleration protein						↑	
egx06a11	At4g10250	22.0 kDa small heat shock protein*				↑			
egx25g03	At5g12020	17.6 kDa class II heat shock protein				↑			
egx18g03	At5g52640	81 kDa heat shock protein*				↑			
eej1c.pk002.f18	At5g56000	Heat shock protein 81-4 (HSP81-4)			↑				
egx18d10	At5g58070	Temperature stress-induced lipocalin*						↑	
(H) Hormone-related									
eej1c.pk006.p21	At1g46768	ERF/AP2 transcription factor family member	TF						↑
eej1c.pk001.d7	At1g53910	Similar to AP2 domain-containing protein	TF	↑					
egx23e06	At1g56220	Dormancy/auxin associated family protein*		↑					
eef1c.pk002.l18	At1g60750	Auxin-induced aldo reductase family member		↑					
eef1c.pk005.a16	At1g66340	Ethylene receptor 1 (ETR1)						↑	
eef1c.pk001.a9	At1g74670	Gibberellin-regulated protein 4 (GASA4)*						↑	
eea1c.pk006.o13	At2g04160	Auxin-induced subtilisin-like serine protease*						↑	
eej1c.pk007.g5	At3g02885	Gibberellin-regulated protein 5 (GASA5)						↑	
egx20b09	At3g14230	ERF/AP2 transcription factor family member	TF	↑					
egx22d11	At3g15730	ABA-mediated stomatal movement regulator						↑	
egx18a08	At4g27410	NAC TF in ABA-mediated dehydration response	TF/stress						↑
eef1c.pk003.c18	At5g42650	Cytochrome p450 in the JA biosynthetic pathway*				↑			
eej1c.pk003.o24	At5g56030	Auxin-induced heat shock protein 90*	Stress		↑				

* Circadianly regulated in *Arabidopsis* (Covington and Harmer, 2007).

[a] At no. of *Arabidopsis* sequence with best hit to *Eucalyptus* microarray target.

[b] As defined in Coutinho et al. (2003) (GT = glycosyl transferase, GH = glycosyl hydrolase, CE = carbohydrate esterase).

[c] Additional characterization for genes falling within multiple categories (TF = transcription factor).

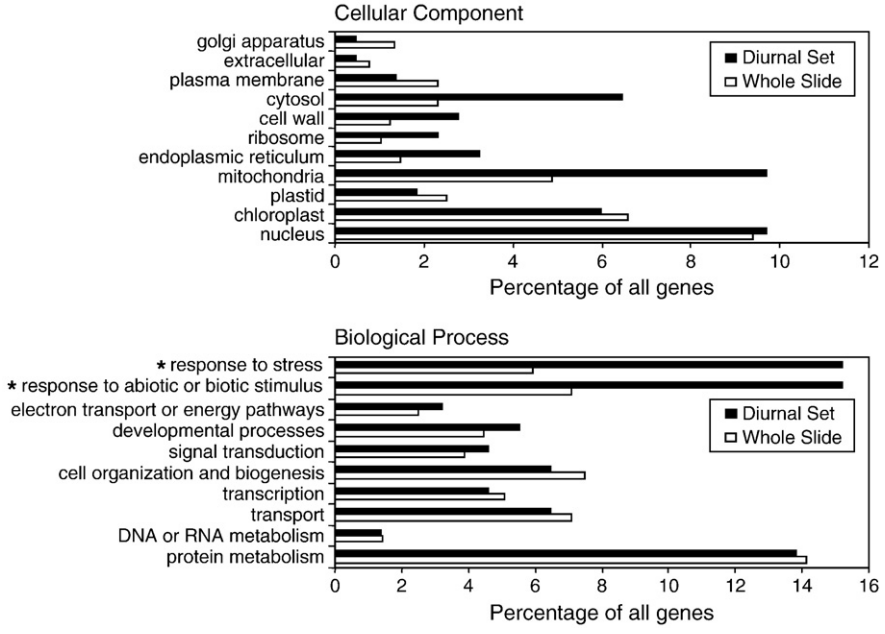

Fig. 3. Gene ontology categories represented on the microarray. Percentage of genes in each category is contrasted for all genes on the microarray slide (white bars) and for the diurnally responsive set of 217 microarray targets (black bars). Several very broad classes including 'other cellular components,' and 'other molecular functions' were removed to enhance clarity. Categories showing the largest proportional changes are at the top of each graph. *Significant at $P = 1.2 \times 10^{-4}$.

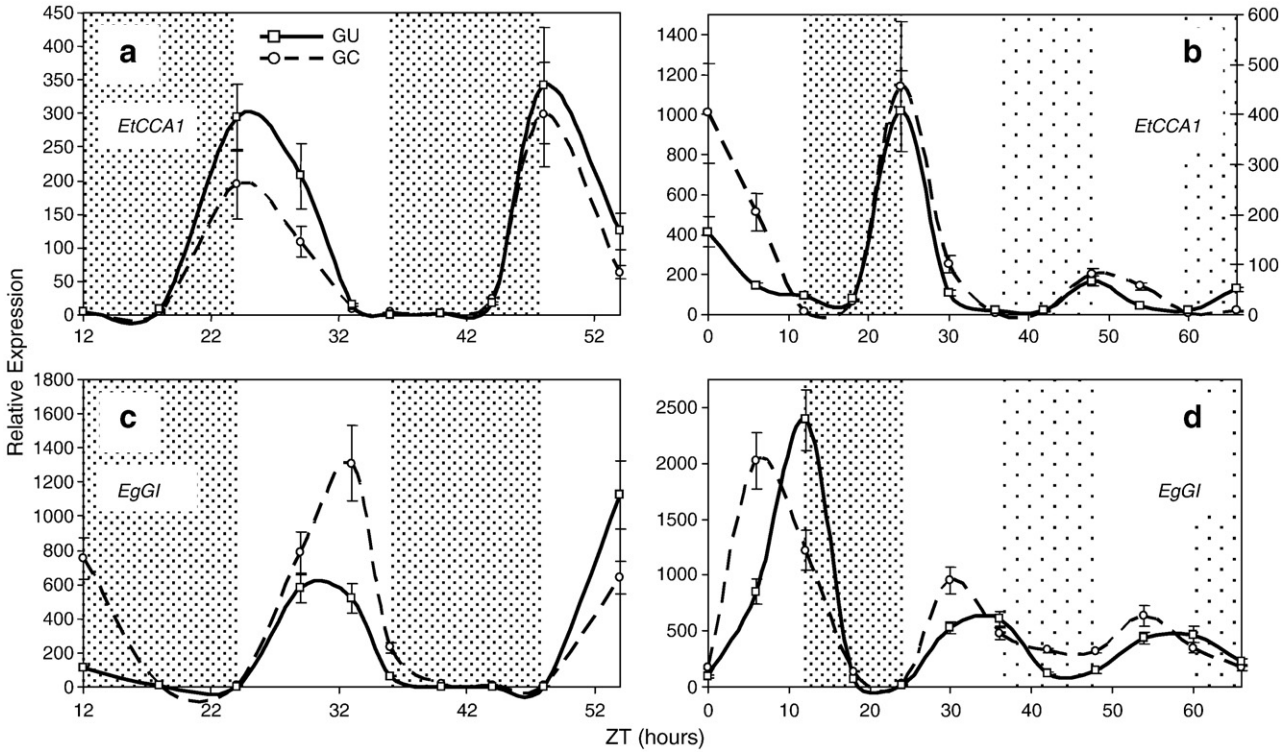

Fig. 4. qRT-PCR expression profiling of two central clock genes in *Eucalyptus* xylem. Relative expression (*Y*-axis) is the ratio of the mean expression level of the gene to the geometric mean of expression of three control genes. Heavily shaded areas indicate night time and unshaded areas day time during light/dark conditions (LD). Lightly shaded areas indicate subjective night during continuous light (LL) conditions. Standard error bars represent the standard error of the mean (SEM) across the three replicate reactions. (a) *EtCCA1* (*E. tereticornis Circadian Clock Associated 1*) expression in immature xylem samples from field-grown trees. (b) *EtCCA1* expression in immature xylem from potted ramets. First 24 h are LD, next 42 h are LL. Primary *Y*-axis = expression in GC, secondary *Y*-axis = expression in GU. (c) *EgGI* (*E. grandis Gigantea*) expression in immature xylem samples from field-grown trees. (d) *EgGI* expression in potted ramets under controlled light. The *X*-axis shows Zeitgeber Time, or hours since dawn.

on each pathway. Diurnally affected genes were observed in carbohydrate metabolism pathways centred on starch and sucrose metabolism (Fig. 5). Genes showing high midday expression included β-amylase (At3g23920) and pyruvate kinase (At3g52990). Sucrose synthase (At5g20830) and UDP-glucuronic acid decarboxylase (At2g28760) showed diurnal profiles with night-time peaks. High morning expression was most clearly seen in pectinase (At1g19170) and xyloglucan endo-transglycosylase (At1g14720) which peaked from night time until early morning.

Co-regulation of diurnal expression was evident in key lignin biosynthesis genes (Fig. 6), as previously reported in *Arabidopsis* (Harmer et al., 2000). Five lignin biosynthetic genes (Table 1) present on our microarray showed similar transcript abundance profiles, with daily fluctuations in mRNA abundance of up to ten fold (fold changes not shown). Two major peaks in expression occurred in this cluster, the first in the hours before dawn and the second during the mid-afternoon.

3.6. Diurnal and circadian regulation of cellulose synthase genes

Diurnal variation in mRNA levels was profiled for all seven known *Eucalyptus* cellulose synthase (*CesA*) genes (Ranik and Myburg, 2006) in field-grown and controlled light samples from the GU and GC genotypes (Fig. S4). Temporal variation in expression was evident, with all *CesA* genes showing two to five fold changes from lowest to peak expression over 24 h in the field-grown samples. *CesA* genes showed their highest fold changes in the controlled light samples, possibly as superimposition of diurnal rhythms may result in reduced circadian signal in field-grown plants (Michael et al., 2008). The large changes in expression level seen in the *CesA* genes did not follow a recurring diurnal profile (Fig. S4). One exception was *EgCesA6* which peaked at dawn or dusk (GC and GU genotypes respectively).

Temporal co-regulation was observed in the secondary cell wall related *CesA* genes (*EgCesA1-3*) whose expression profiles were highly correlated within experiments (avg $r^2 = 0.73$ in GU, 0.92 in GC, Table 2). In contrast, the primary cell wall related *CesA* genes (*EgCesA4-7*) did not share similar expression profiles (avg $r^2 = 0.22$ in GU, 0.31 in GC, Table 2), and correlation between primary and secondary cell wall related *CesA* genes was also low (avg $r^2 = 0.20$ in GU, 0.24 in GC, Table 2). *CesA* genes showed genotype-specific expression profiles, with the average r^2 value of correlation for each *CesA* gene in GU and GC only 0.07 (results not shown). Growth in controlled light conditions revealed that *EgCesA6* may be under circadian control, with regularly timed peaks in expression under constant light. Interestingly, *EgCesA6* expression profiles were in opposing phases in the GU and GC genotypes (Fig. S5).

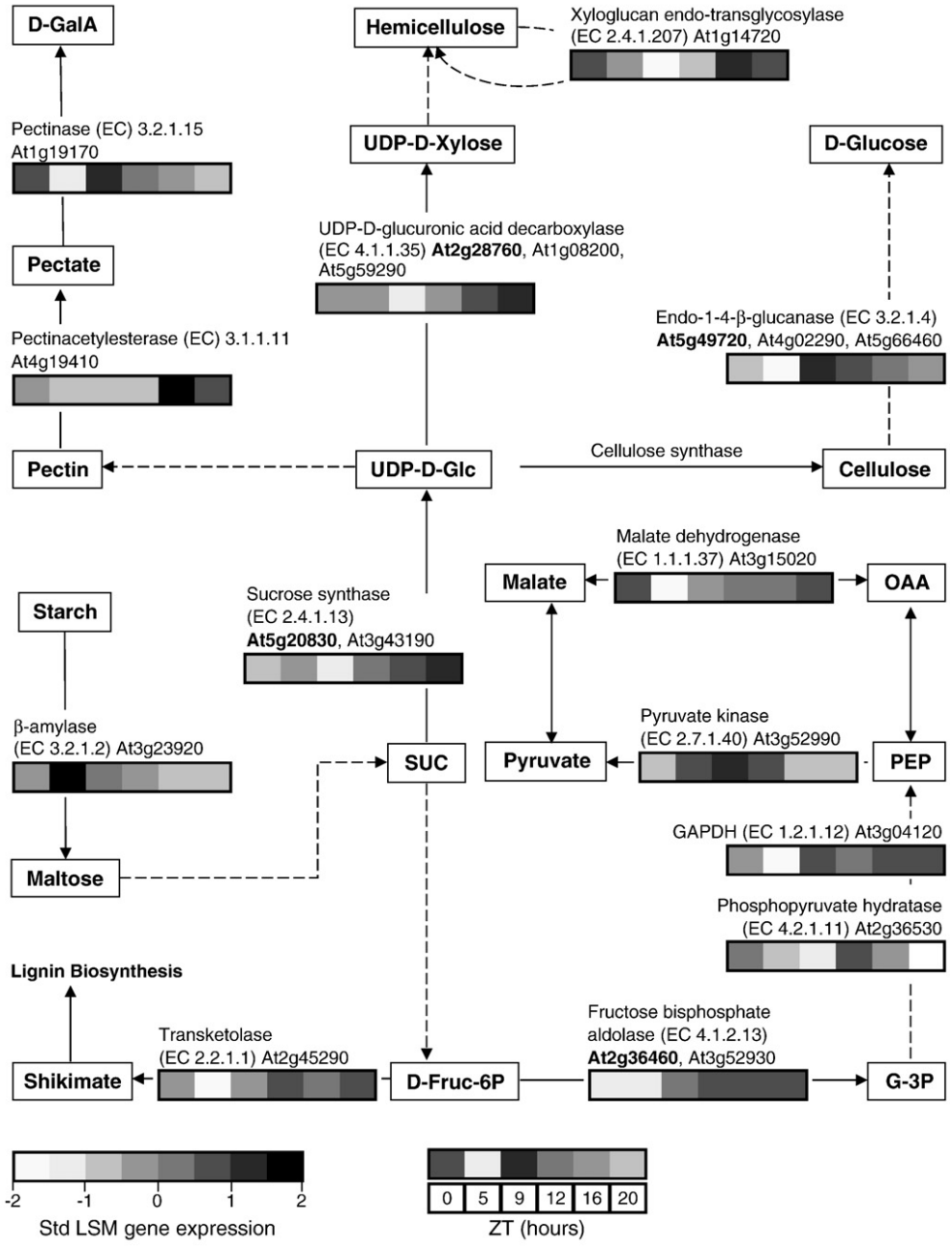

Fig. 5. Metabolic pathway analyses of diurnally influenced carbohydrate metabolism genes in field-grown *Eucalyptus* trees. Pathways are adapted from Aracyc (www.arabidopsis.org/tools/aracyc/) and KEGG Pathway (www.genome.ad.jp/kegg/). Solid arrows indicate reactions occurring in a single step, dashed arrows indicate that intermediate steps have been omitted for clarity. Changes in transcript abundance are represented on a greyscale continuum of standardised least square means estimates of gene expression, where white represents low expression and black, high expression. ZT = Zeitgeber Time, or hours since dawn. Gene expression is shown for six sampled time points, namely 06:00 (ZT 0); 11:00 (ZT 5); 15:00 (ZT 9); 18:00 (ZT 12); 22:00 (ZT 16) and 02:00 (ZT 20). Abbreviations: D-GalA = D-galacturonate; UDP-D-Glc = UDP-D-glucose; OAA = oxaloacetate; SUC = sucrose; PEP = phosphoenolpyruvate; D-Fruc-6P = D-fructose-6P; G-3P = glyceraldehyde-3P; and GAPDH = glyceraldehyde 3-phosphate dehydrogenase. At numbers and corresponding EC (Enzyme Commission) numbers are given for each step in the pathway represented by a diurnally influenced microarray target. In some cases more than one microarray target (At number) corresponded to a single EC number. In these cases a representative expression profile (At number in bold) is illustrated.

4. Discussion

4.1. The central circadian oscillator is active in wood-forming tissues of Eucalyptus and in phase with that of Arabidopsis

Recent studies in *Arabidopsis* have proposed a model for the central oscillator of the circadian clock consisting of multiple, interacting feedback loops (Locke et al., 2006; Zeilinger et al., 2006). These feedback loops result in oppositely phased gene expression profiles for *CCA1* and *GI*. We have found that the expression profiles of these central clock genes in *Eucalyptus* xylem (Table 1; Fig. 4) follow the phases of their *Arabidopsis* counterparts, as has been illustrated in chestnut (Ramos et al., 2005). It appears, therefore,

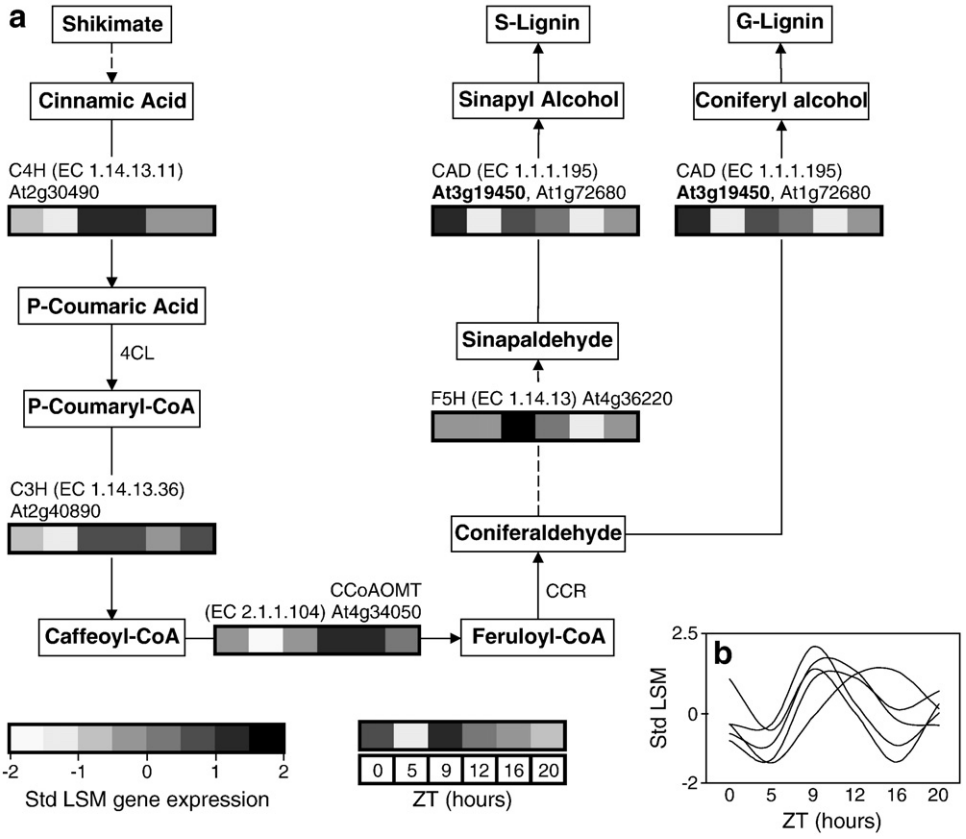

Fig. 6. Microarray analysis reveals coordinated diurnal variation of gene expression in the lignin biosynthetic pathway in field-grown *Eucalyptus* trees. (a). Pathway adapted from KEGG Pathway (www.genome.ad.jp/kegg/). Changes in transcript abundance are represented on a greyscale continuum of standardised least square means estimates of gene expression, where white represents low expression and black, high. ZT = Zeitgeber Time, or hours since dawn. Gene expression is shown for six sampled time points, namely 06:00 (ZT 0), 11:00 (ZT 5), 15:00 (ZT 9), 18:00 (ZT 12), 22:00 (ZT 16) and 02:00 (ZT 20). Abbreviations: C4H = cinnamate-4-hydroxylase; CAD = cinnamyl alcohol dehydrogenase; 4CL = 4-coumarate:CoA ligase; F5H = ferulate-5-hydroxylase; C3H = cinnamate-3-hydroxylase; CCoAOMT = caffeoyl-CoA *O*-methyltransferase; and CCR = cinnamoyl-CoA reductase. (b) Temporal co-expression of microarray targets related to lignin biosynthesis. *Arabidopsis* homologues represented are: CCoAOMT (At4g34050), C4H (At2g30490), C3H (At2g40890), CAD (At3g19450) and F5H (At4g36220).

that the central oscillator is conserved across herbaceous annuals and woody perennials.

ZTL maintains robust cycling of the clock by targeting the TOC1 protein for degradation (Mas et al., 2003). *ZTL* is constitutively expressed in *Arabidopsis*, but displays circadian fluctuation at the protein level (Kevei et al., 2006), conferred by protein binding and regulation via the central clock protein GI (Kim et al., 2007). Our microarray results showed that *EtZTL* mRNA oscillates in *Eucalyptus* with a large fold change (26-fold) and a phase similar to that of *EgGI* (Fig. S3). These results suggest that unlike in *Arabidopsis*, *Eucalyptus ZTL* is controlled at the transcriptional level, as previously reported in *Mesembryanthemum crystallinum* (Boxall et al., 2005).

Table 2
Regression values (r^2) for pair-wise comparisons of diurnal transcript abundance profiles of the seven *Eucalyptus CesA* genes with comparisons among the secondary cell wall *CesA* genes shown in bold.

GU[a] / GC[a]	CesA1	CesA2	CesA3	CesA4	CesA5	CesA6	CesA7
CesA1		**0.65**	**0.71**	0.26	0.18	0.18	0.11
CesA2	**0.92**		**0.84**	0.32	0.12	0.05	0.43
CesA3	**0.91**	**0.92**		0.26	0.10	0.06	0.32
CesA4	0.19	0.18	0.25		0.28	0.26	0.29
CesA5	0.28	0.35	0.31	0.33		0.34	0.08
CesA6	0.13	0.04	0.13	0.32	0.31		0.09
CesA7	0.32	0.40	0.29	0.19	0.55	0.15	

[a]Values for comparisons within GU (above diagonal) or GC (below diagonal), averages of controlled light and field-grown samples.

4.2. Carbon allocation pathways exhibit diurnal regulation in *Eucalyptus* trees

Carbon levels in plants are known to fluctuate between surplus during the daytime hours and deficit during the night (Stitt et al., 2007). Temporal variation in carbon levels is compounded by the spatial separation of carbon source and sink tissues in large woody plants. The daily coordination of carbon allocation is not well described in trees, which differs from herbaceous model species in their long life spans, and large-scale production and assimilation of photosynthate. Diurnal control of gene expression in wood-forming tissues

may be an important approach to coordinating xylogenesis with daily fluctuation in carbon availability in trees (Ramos et al., 2005).

Carbon captured during photosynthesis can be used in the originating source tissue, stored for later use, or transported to sink tissues for a variety of uses. Regulating the allocation of carbon to these ends is necessary for optimal plant growth under changing environmental conditions (Smith and Stitt, 2007) such as those experienced during the diurnal cycle. To ensure continued provision of carbon after dark, carbohydrate stores that were set aside during the day need to be mobilised. The plant must therefore sense sugar levels throughout the day and store enough carbon to sustain growth and metabolism during the night. The circadian clock may play an important role in coordinating these carbon allocation processes on a daily basis (Weise et al., 2006; Covington and Harmer, 2007).

Trehalose-6-P (Tre6P) has been suggested to signal changing carbon levels (Stitt et al., 2007), as rising sugar levels, accompanied by increases in Tre6P stimulate starch biosynthesis. We found two genes with predicted Tre6P synthase (TPS) protein domains that exhibited diurnal variation in *Eucalyptus* (Table 1). The *Eucalyptus TPS* genes showed two peaks in expression, one at the end of the night/early morning, and a second during the mid-afternoon (transcript abundance profiles for these genes, and those discussed below are provided in Table S2). High mid-afternoon expression of *TPS* could serve to raise levels of Tre6P, resulting in increased starch synthesis during the daylight hours when sugars are abundant. In *Arabidopsis*, *TPS* is circadianly regulated with strong expression during daylight hours, and a mid-afternoon peak (Harmer et al., 2000).

Five genes involved in the energy releasing glycolytic pathway were found to be diurnally influenced (Fig. 5). These diurnal changes may modulate carbon flow into cellular energy metabolism at appropriate times of day. Three genes are involved in synthesising phosphoenolpyruvate for entry into the TCA cycle including fructose bisphosphate aldolase, phosphopyruvate hydratase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Table 1). The three genes appeared to be coordinately regulated in *Eucalyptus* xylem with peak transcript abundance occurring at night.

Most sugar molecules produced during the day are temporarily stored in the form of starch until required (Stitt et al., 2007). Several genes responsible for starch degradation and mobilisation are under circadian control in *Arabidopsis* (Lu et al., 2005). This generates free sugars, primarily maltose, for growth and metabolism when required by the plant. We found increased expression of β-amylase, which hydrolyses starch to maltose, showed highest expression at 11.00 (ZT5, Fig.5). The expression profile of this gene in *Eucalyptus* differed from its circadianly controlled *Arabidopsis* homologue, which peaks almost 12 h later at around 02:00 (ZT 16, Covington and Harmer, 2007). A midday peak of this gene may be related to the role of maltose in protecting proteins and membranes under temperature stress, as this specific gene has been shown to be induced by heat shock in *Arabidopsis* (Kaplan and Guy, 2004).

4.3. Cell wall formation genes show diurnal expression patterns in Eucalyptus xylem

Carbon molecules that are transported to differentiating secondary xylem cells are primarily allocated to one of the three major cell wall components: cellulose, hemicellulose and lignin. Genes encoding several key enzymes related to these biosynthetic pathways showed diurnally responsive expression profiles in *Eucalyptus* and may be circadianly controlled, as are many *Arabidopsis* cell wall formation genes (Harmer et al., 2000; Rogers et al., 2005).

Sucrose synthase (SuSy, or SUS) is highly upregulated in cellulose-rich tissues (Andersson-Gunnerås et al., 2006) where it converts sucrose to fructose and UDP-glucose. UDP-glucose is the substrate of CESA proteins for cellulose production. We found two *Eucalyptus SuSy* genes (Table 1) that exhibited peak expression at 02:00, (ZT 20, Fig. 5). This may be associated with increased night-time production of cellulose and hemicellulose in *Eucalyptus* xylem.

SuSy proteins exist in two main isoforms; a soluble form involved in general cellular metabolism (S-SuSy), and a particulate form (P-SuSy) associated with the plasma membrane and linked to cellulose biosynthesis (Haigler et al., 2001). The particulate nature and activity of SuSy is affected by Ca^{2+} levels in the cell, with high Ca^{2+} concentrations favouring the P-SuSy form. Cellular Ca^{2+} levels are circadianly regulated in *Arabidopsis* (Love et al., 2004) and are hypothesised to modulate various stimulus-induced signalling events (Dodd et al., 2005a,b, 2006). We found three genes related to calcium signalling with diurnal expression profiles in *Eucalyptus* xylem (Table 1), two of which peaked near midnight. Increased Ca^{2+} levels occurring simultaneously with high SuSy transcript abundance could indicate post-transcriptional control of P-SuSy activity and may be associated with elevated cellulose deposition during the night.

A key enzyme in the production of hemicellulose, UDP-D-glucuronic acid decarboxylase (Table 1) shared a similar transcript abundance profile with *SuSy* in our study, peaking at ZT 20 (02:00, Fig. 5). UDP-D-glucuronic acid decarboxylase channels UDP-glucuronic acid into the biosynthesis of xylan, the most abundant hemicellulose in angiosperm wood. The coordinated production of hemicellulose and cellulose in woody tissues could be advantageous to the tree, as xylan may function as a cross-linking matrix holding the cellulose microfibrils together (Hayashi, 1989). Diurnal differences in the supply of hemicellulose components to the cell wall have previously been noted in the conifer *Cryptomeria japonica* (Hosoo et al., 2006), where hemicelluloses are supplied to the secondary cell wall at highest levels during the dark period.

The expression profiles of the seven cloned *Eucalyptus CesA* genes showed large diurnal changes in expression levels, consistent with the hypothesis that cellulose production is temporally regulated (Fig. S4). However, the timing of expression peaks was largely irregular over the three days examined, and *CesA* transcript abundance patterns differed between field-grown and controlled light trees, as well as between clonal genotypes. It is possible that timing of cellulose production in

Eucalyptus may be primarily controlled at the CESA protein level and/or at the level of *SuSy* expression and localization. Strong temporal co-regulation of the secondary cell wall related *CesA* genes (Table 2) does suggest that temporal transcriptional regulation of *CesA* genes is important in *Eucalyptus*. We recently identified putative light responsive promoter elements in both primary and secondary cell wall related *CesA* genes (Creux et al., 2008).

The only *CesA* gene that exhibited a regular and possibly circadian expression profile was *EgCesA6* (Fig. S4). *EgCesA6* is closely related to *AtCesA6* (Ranik and Myburg, 2006) which has previously been noted to exhibit interaction with light levels (Fagard et al., 2000). This is hypothesised to be due to phytochrome controlling the expression of one or more CESA isoforms, suggesting a connection between light, the circadian clock and at least one of the *CesA* genes. The causal factor behind the opposite phasing of *EgCesA6* in the GU and GC genotypes (Fig. S5) is currently unknown.

4.4. Lignin biosynthetic pathway: two diurnal peaks indicate roles in UV protection and growth

Lignin is the second most abundant component of secondary cell walls. It is responsible for cell wall reinforcement, impermeability to water and resistance to microbial attack (Boerjan et al., 2003). Due to the importance of lignin in the plant cell wall, and the large amount of carbon required for its synthesis, lignin deposition is regulated both spatially and temporally (Rogers and Campbell, 2004). Many of the genes involved in lignin biosynthesis are circadianly regulated in *Arabidopsis*, peaking before dawn (Rogers et al., 2005). Temporal regulation of the phenylpropanoid pathway has been suggested to facilitate daily production of a UV protective 'phenolic sunscreen,' and to temporally separate cell growth from lignin deposition, as lignin deposition limits further cell expansion.

Our results are consistent with both hypotheses. *Eucalyptus* genes in the lignin biosynthetic pathway showed two expression peaks, as in *Arabidopsis* (Fig.6). It has been suggested that the night-time peak in *Arabidopsis* is controlled by the central oscillator, while the day time peak is affected by light levels (Rogers et al., 2005). The first peak in expression in *Eucalyptus* occurred in the mid-afternoon (ZT 9), when phenolics may be required for UV protection. The second peak was observed during the hours preceding dawn (ZT 20–24), possibly when cell expansion has ceased, allowing time for the reinforcement of new cell walls.

4.5. Xylem cell expansion and secondary wall formation may be coordinated diurnally in Eucalyptus

The upregulation of *Eucalyptus* genes involved in the allocation of carbohydrate precursors to cell wall polymers during the night could be indicative of a temporal coordination of cell expansion and secondary cell wall thickening and lignification. This temporal coordination could regulate lignification and cell wall thickening to occur after cellular expansion has taken place, in order to reinforce newly enlarged cells. Temporal and spatial separation of cell growth and reinforcement (Harmer et al., 2000) may be important in woody plants where vessel and fibre precursors undergo massive expansion, and lateral growth supports large increases in tree volume and weight.

The influx of water into plant cells necessary for cellular expansion (Uehlein and Kaldenhoff, 2006) is aided by membrane bound aquaporins which facilitate the transport of water into and out of plant cells. We found two diurnally expressed aquaporins in *Eucalyptus* (Table 1). δ-TIP (Delta Tonoplast Intrinsic Protein) had expression peaks at 06:00 and 22:00, while plasma membrane intrinsic protein 2B (PIP2B) peaked at 15:00. PIP2B is circadianly regulated in *Arabidopsis* (Harmer et al., 2000; Covington and Harmer, 2007) and may be under similar control in *Eucalyptus*. The night-time peak in δ-TIP expression levels may be associated with cell expansion during the early night as has been suggested to occur in *Arabidopsis* (Harmer et al., 2000).

4.6. Diurnal cycling of transcription factors and hormone related genes may have widespread effects on affected pathways

The discovery of diurnally cycling genes in *Eucalyptus* xylem is a first step towards uncovering which developmental and metabolic processes may be under temporal control in trees. Some diurnally influenced genes, due to their widespread downstream effects may impart temporal rhythm on whole pathways. These include transcription factors (TFs), hormone synthesis and signalling pathway genes, which could provide links between the pathways that they control and the central clock.

Eleven TFs were found to be diurnally influenced in *Eucalyptus* xylem (Table 1), including ATMYB20, a *MYB* gene family member that is known to regulate the biosynthesis of monolignols (Rogers and Campbell, 2004), and is specifically related to wood formation. ATMYB20 is associated with fibre formation, and is regulated by SND1, a key regulator of TFs involved in secondary cell wall biosynthesis (Zhou et al., 2006). In *Eucalyptus* xylem, ATMYB20 expression was highest in the second half of the day, coinciding with the peak expression of several of the cell wall biosynthesis genes on our microarray, and may be implicated in their regulation.

Several of the diurnally responsive TFs were involved in stress-related pathways, including ATWRKY38, a WRKY family member associated with stress response (Mare et al., 2004) and *Arabidopsis thaliana* Homeobox 7 (ATHB7). ATHB7 is expressed during drought stress (Engström et al., 1996), and showed peak expression at midday in *Eucalyptus* xylem, possibly in anticipation of increased water stress at this time. In total, we found 24 diurnally influenced stress-related genes in *Eucalyptus* (Table 1), which may be involved in anticipating and preparing for cyclic stress. The diurnally affected *Eucalyptus* heat shock proteins all showed similar expression profiles, peaking around midday (Table 1). Clock control of heat shock genes could direct accumulation of heat shock proteins in trees in anticipation of high temperatures, as in *Arabidopsis*, where approximately 50%

of all heat responsive genes were clock regulated (Covington et al., 2008).

Many plant hormone synthesis and signalling pathway components are clock regulated in *Arabidopsis* (Covington and Harmer, 2007), conferring temporal control over the pathways that they control. Hormone pathways related to wood formation, including auxin (Demura and Fukuda, 2007) and gibberellic acid (GA) (Björklund et al., 2007) had genes with diurnal expression profiles in *Eucalyptus*. We identified four auxin-related genes that were diurnally responsive (Table 1), three of which are circadianly regulated in *Arabidopsis* (Covington and Harmer, 2007). Gibberellin-regulated protein 4 (GASA4) and GASA5 were also diurnally influenced in *Eucalyptus*, both peaking at ZT 16 (22:00).

Four ethylene-related genes including three members of the ethylene response factor (ERF)/AP2 transcription family exhibited diurnal expression profiles in *Eucalyptus* (Table 1). The *ERF/AP2* genes were temporally co-expressed, with their highest expression at ZT 0. The ERF domain has specific DNA binding activity to the GCC box, a sequence element involved in ethylene-responsive gene transcription (Mitsuda et al., 2007). Increased transcription of these genes before dawn could indicate temporal regulation or gating of ethylene sensitivity in woody tissues. Ethylene participates in a wide range of physiological activities including plant growth and stress response (Bleecker and Kende, 2000).

In conclusion, we showed that diurnal cycles influence gene expression in differentiating *Eucalyptus* xylem, a very strong sink tissue, where they may play a role in the coordination of carbon allocation and cell wall formation. Furthermore, our results suggest that the central oscillator of the plant circadian clock is active in the tree stem, where it is likely to be responsible for some of the observed diurnal changes and may regulate many of the biological processes discussed above. Diurnally influenced genes are involved in a wide variety of metabolic tasks, including the allocation of carbon to various end uses such as formation of secondary cell walls.

The number of genes found to be diurnally regulated in our study is fairly low compared to *Arabidopsis*, where almost 90% of genes cycle in transcript abundance over a wide range of photo- and thermocycles (Michael et al., 2008). This is probably due to the fact that the tissue we examined is not a green tissue exposed to fluctuating light levels. Additionally, we studied a single tissue made up of few cell types, rather than whole plants. Finally, our ability to detect small changes in gene expression was limited by using field-grown trees under natural conditions. This final factor will most likely have resulted in us underestimating to some degree the number of diurnally influenced genes in developing xylem tissue.

Our results were obtained from 2608 cDNAs representing ~10% of the expected gene number in *Eucalyptus*. The *Eucalyptus* genome is scheduled to be the second forest tree genome sequenced (www.eucagen.org). A whole-transcriptome *Eucalyptus* microarray will allow more complete determination of diurnal and circadian gene regulation in this tree. The limitations of microarray profiling will continue to be relevant, however, including the fact that changes in transcript abundance may lead to smaller or delayed changes in protein activity (Gibon et al., 2004; Blasing et al., 2005). Microarray analysis is furthermore unable to detect diurnal regulation at the post-translational level. Despite the limitations of this study and microarray analysis in general, our results represent a positive step towards a more detailed understanding of the temporal control of gene expression and its effect on carbon allocation in wood-forming tissues. This level of understanding will be important for future efforts to adapt fast-growing plantation trees for increased biomass and bioenergy production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2010.02.087.

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